Cell death features induced in *Leishmania major* by 1,3,4-thiadiazole derivatives

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**Highlights**

- Thiazole analogs induced loss of plasma membrane integrity, DNA breakage and proteolysis of PARP.
- These analogs were shown to induce necrosis on *Leishmania major* promastigotes.
- These analogs decreased acid phosphatase activity significantly during virulent growth stage of promastigotes.

**ABSTRACT**

Under a variety of stress conditions, *Leishmania* species display some morphological and biochemical features characteristic of mammalian programmed cell death or necrosis. Nitroheteraryl-1,3,4-thiadiazoles induce cell death in *Leishmania major* (*L. major*). Putative mechanisms of action of these compounds were investigated in vitro at cellular and molecular levels. We used colorimetric assay to measure acid phosphatase activity which is an indicator of cell viability in the promastigotes. The mode of toxicity was determined by detection of phosphatidylserine translocation to the surface, evaluation of cell membrane integrity, and *in situ* dUTP nick end-labeling assay. We also determined poly-ADP-ribose polymerase-like protein (PARP) level in the parasites after treatment.

A significant reduction of acid phosphatase level, one of the most crucial and virulent factors of the parasite was found in parasites treated with 1,3,4-thiadiazole derivatives. In addition, 1,3,4-thiadiazole derivatives induced loss of plasma membrane integrity, DNA breakage, proteolysis of PARP and necrotic-like death in the parasites.

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**1. Introduction**

About two million new cases of human leishmaniasis are reported annually and about 350 million people are living in the endemic areas (Sundar and Chatterjee, 2006; Vannier-Santos et al., 2002). The global burden of this parasitic disease is further stressed out, due to the changing patterns of the disease, increasing numbers of human immunodeficiency virus (HIV) co-infections and the appearance of resistant strains. In addition, human migration to the area where leishmaniasis is zoontic makes its resurgence a possibility (Bailey and Lockwood, 2007; Olivier et al., 2003; Pasqua et al., 2005).

The current anti-parasitic chemotherapy is expensive, has undesirable side effects or, in many patients, is only marginally effective; therefore finding new targets for chemotherapy seems...
very crucial (Croft and Yardley, 2002; Ouellette et al., 2004; Werbovetz, 2000). Well-designed synthetic crescent-shaped heterocyclic compounds have displayed excellent activity against eukaryotic parasites such as *Trypanosoma* and *Leishmania*. These compounds rapidly enter the unicellular parasites, concentrate in the kinetoplast and nucleus, and aiming mitochondrial and genomic DNA damage. These genotoxic agents cause severe DNA strand breaks and stimulate sensor enzymes, PARP which contribute to program cell death (Bailly, 2000; Palchaudhuri and Hergenrother, 2007; Pommier et al., 1998; Tentori and Graziani, 2005; Wilson et al., 2008).

Our previous study indicated that the piperazine moiety of 2-(nitroaryl)-1,3,4-thiadiazoles bearing a piperazine-1-yl substituent at the C-5 position of 1,3,4-thiadiazole nucleus possesses enough structural flexibility. Therefore N-4 substituent of 1-[5-nitroaryl)-1,3,4-thiadiazol-2-yl]piperazines were synthesized and antileishmanial activity of nitroheteroaryl-1,3,4-thiadiazole-based compounds on both promastigote and amastigote forms of *L. major* strain (MRHO/IR/75/ER) studied. We also measured the selectivity and efficiency of those compounds against different wild types of *Leishmania* and their intracellular forms as well as their toxicity on host cells and inhibition of topoisomerases I and II activity (Behrouzi-Fardmoghadam et al., 2008; Poorrajab et al., 2009a,b).

It has been shown that *Leishmania* parasite uses surface apoptotic marker phosphatidylserine as a Trojan horse for silent entry into professional-phagocytic host cells which result in down-regulation of the pro-inflammatory cytokines (Arnoult et al., 2002; Lassey et al., 2008; Sen et al., 2004; van et al., 2006; Wanderley et al., 2006; Zangger et al., 2002). In contrast, induction of a necrosis-like death in a pathogen has a benefit for stimulation of immune system to eliminating invasive parasite (Arnoult et al., 2002; Elmore, 2007; Festjens et al., 2006; Zangger et al., 2002).

The aim of the present work was to clarify the nitroheteroaryl-1,3,4-thiadiazole derivatives toxicity on the different growth phase of *Leishmania* promastigotes and the mechanism(s) involved in the parasite killing.

### 2. Materials and methods

#### 2.1. Test compounds

All tested nitroheteroaryl-1,3,4-thiadiazole derivatives (Fig. 1) were lab-made compounds. The synthesis details, characterization, IC₅₀ determination, and topoisomerase inhibition activity of the compounds have been previously described (Behrouzi-Fardmoghadam et al., 2008; Poorrajab et al., 2009a,b).

#### 2.2. Promastigote culture

Promastigotes of *L. major* strain (MRHO/IR/75/ER) were cultured as described previously (Behrouzi-Fardmoghadam et al., 2008; Poorrajab et al., 2009a, 2009b). Briefly, 2 x 10⁶ cells/ml were routinely inoculated and cultured in RPMI 1640 medium (pH 7.2, containing 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, UK), enriched with 10% heat-inactivated fetal calf serum, at 25 °C. Based on growth curve, logarithmic phase appears at 48 h and stationary phase appears at 96 h (data not shown).

#### 2.3. Treatment of promastigotes with the compounds

For drug treatment, IC₅₀ doses of selected compounds as indicated in Table 1 were added to the logarithmic or stationary phase of the parasites.

Drug solutions were made in 2% DMSO as stock and diluted to make appropriate concentrations in the medium immediately before adding to the parasite culture. 200 μl of 4 x 10⁵ promastigotes in medium with or without compounds was dispensed in microplate wells and incubated for 24 h at 25 °C. In order to show the specific effects of the compounds, we used 60 x 10⁸ μM glucan-time which is its IC₄₀ on *L. major* promastigotes since it is used to treat cutaneous and visceral leishmaniasis in humans (Rocha et al., 2011). Also 2% DMSO was used as vehicle control.

#### 2.4. Acid phosphatase activity assay

Acid phosphatase activity was measured to show the potential impact of the compounds on parasite growth and virulence. The IC₅₀ concentration of compounds was added to the either logarithmic or stationary phases of parasites, incubated for 24 h. Total acid phosphatase activity (secretory, membranous and cytoplasmic) was determined as follows (Aragon et al., 2001; Lee et al., 2007): After 24 h incubation, 20 μl of lysis buffer (1 M sodium acetate, pH 5.5 and 1% Triton X-100) containing 10 mg/ml *p*-nitrophenyl phosphate was directly added to each well. Incubation was continued for a further 6 h at 37 °C, and the production of *p*-nitrophenol was determined by optical density measurements at 405 nm using an ELISA plate reader.

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Fig. 1. Chemical structures of 2,5-disubstituted-1,3,4-thiadiazole derivatives.
100 and incubated with TUNEL reaction mixture containing nucleo-

1.2.5. Determination of mode of drug-mediated cell death in L. major

promastigotes

The percentage of viable, necrotic and apoptotic cells were
determined by flow cytometry (FACScan, LYSISIL, Becton Dickinson,
USA) using surface Annexin V detection and 7-Amino-actinomycin D
(7-AAD) incorporation (all from BD Biosciences, USA). Logarithmic-

phase promastigotes were incubated with 1f, 1a and 2a IC\textsubscript{50}

analog or 2% DMSO for 3 or 6 h. Cells were centrifuged (3000g for
5 mins), washed twice in PBS and resuspended in annexin V
binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl\textsubscript{2}], then. Annexin V–FITC and 7-AAD were added to
the suspension according to the manufacturer's instructions
and the resulted suspension was incubated in dark condition at
20–25 °C for 30 mins. Data acquisition was carried out on a flow
cytometer and analysed using LYSISIL software. If there is an alter-
ation in the membrane integrity (due to externalization of phos-
phatidylserine), annexin V detects both pro- and late-apoptotic
cells. Therefore, the simultaneous addition of 7-AAD, which does
not enter healthy cells with an intact plasma membrane, discrim-


3. Results

3.1. Acid phosphatase activity was significantly decreased after
treatment with Nitroheteroaryl-1,3,4-thiadiazole derivatives in both
stages of L. major growth

L. major contains considerable amounts of acid phosphatase
which is one of the most important factors contributing to the
spread of virulent parasites. Acid phosphatase activity is a reliable
indicator for the parasite growth rate as well as for its virulent
potency (Katakura and Kobayashi, 1988; Olivier et al., 2005). Treat-
ment with nitroheteroaryl-1,3,4-thiadiazoles derivatives signifi-
cantly decreased acid phosphatase activity on both logarithmic
and stationary promastigotes. The impact of the compounds on
acid phosphatase activity was shown to be more profound on
stationary phase (~3.5-folds) compared to logarithmic phase
(~2.5-folds) (Table 1). However, glucantime had smaller effect on
stationary phase comparing with thiadiazole derivatives.

3.2. Nitroheteroaryl-1,3,4-thiadiazole derivatives induced cell death is
associated with nuclear and cytoplasmic feature of necrosis

In order to obtain a clue to the possible effect of nitroheteroaryl-
1,3,4-thiadiazole derivatives against L. major, logarithmic phase
promastigotes were treated with IC\textsubscript{50} compounds or 2% DMSO
and analyzed by flow cytometry. 1f, 1a and 2a compounds used
in this experiment had inhibitory effects on parasite topoisomeras-
es (Top) I and II catalytic activity more than 60% and selectivity
index more than 20 (Poorrajab et al., 2009b). After 3 h incubation,
DMSO-treated promastigotes displayed a homogeneous popula-
tion of living cells, labeled-cell population with Annexin V was
~0.7% and 7-AAD permeability was ~0.4%, whereas in the com-
 pound treated population, a hallmark of necrotic cell death process

Table 1

Acid phosphatase activity assay of L. major promastigotes at two different growth phases, treated with the selected compounds and drug solvent DMSO as vehicle control and glucantime as a selective drug for 24 h.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC\textsubscript{50}(\mu M)\textsuperscript{a}</th>
<th>OD</th>
<th>ACP activity of control sample/ACP activity of drug-treated sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Logarithmic phase</td>
<td>Stationary phase</td>
<td>Logarithmic phase</td>
</tr>
<tr>
<td>1f</td>
<td>9.35 ± 0.67</td>
<td>1.207 ± 0.01</td>
<td>1.844 ± 0.149</td>
</tr>
<tr>
<td>2f</td>
<td>11.75 ± 1.1</td>
<td>1.148 ± 0.027</td>
<td>1.791 ± 0.031</td>
</tr>
<tr>
<td>2g</td>
<td>13.11 ± 0.86</td>
<td>1.347 ± 0.116</td>
<td>1.885 ± 0.001</td>
</tr>
<tr>
<td>2b</td>
<td>10.73 ± 0.69</td>
<td>1.147 ± 0.045</td>
<td>2.1 ± 0.01</td>
</tr>
<tr>
<td>2c</td>
<td>13.19 ± 0.96</td>
<td>1.253 ± 0.095</td>
<td>2.1 ± 0.01</td>
</tr>
<tr>
<td>2a</td>
<td>11.69 ± 1.3</td>
<td>1.106 ± 0.025</td>
<td>2.1 ± 0.01</td>
</tr>
<tr>
<td>1a</td>
<td>10.39 ± 0.95</td>
<td>1.184 ± 0.024</td>
<td>2.1 ± 0.01</td>
</tr>
<tr>
<td>Glucantime\textsuperscript{b}</td>
<td>(60 ± 2.82) × 10\textsuperscript{3}</td>
<td>1.229 ± 0.414</td>
<td>2.82 ± 0.18</td>
</tr>
<tr>
<td>DMSO</td>
<td>–</td>
<td>2.545 ± 0.075</td>
<td>5.67 ± 0.58</td>
</tr>
</tbody>
</table>

AcP: Acid phosphatase.

Data represent mean ± standard deviation of three different experiments.

\textsuperscript{a} IC\textsubscript{50}s were previously determined for L. major promastigotes of vaccine strain (Behrouzi-Fardmoghadam et al., 2008).

\textsuperscript{b} Using increasing concentrations of Glucantime (60 ± 2.82)\times 10\textsuperscript{3} \mu M showed 40% growth inhibition.
Table 2

Percent of Annexin V + and 7-AAD + L. major promastigotes treated with IC50 concentration of the Nitroheteroaryl-1,3,4-thiadiazole analogs after 3 h.

| Compounds | 3 h treatment | | | |
|-----------|---------------|-----------------|-----------------|
|            | Annexin V    | 7-ADD | Ann. V + 7-AAD | Without staining |
| DMSO       | 0.7 ± 0.05    | 0.4 ± 0.1      | 0.1 ± 0.17      | 98.6 ± 0.4       |
| 1f         | 1.7 ± 0.1     | 6.4 ± 0.15     | 1.3 ± 0.08      | 90.2 ± 0.5       |
| 1a         | 1.8 ± 0.2     | 9.3 ± 0.07     | 1.4 ± 0.15      | 87.5 ± 0.7       |
| 2a         | 1.9 ± 0.09    | 11.4 ± 0.1     | 1.6 ± 0.1       | 85 ± 0.18        |

Table 3

Percent of Annexin V and 7-AAD labeling in L. major promastigotes treated with IC50 concentration of the Nitroheteroaryl-1,3,4-thiadiazole analogs after 6 h.

| Compounds | 6 h treatment | | | |
|-----------|---------------|-----------------|-----------------|
|            | Annexin V    | 7-ADD | Ann. V + 7-AAD | Without staining |
| DMSO       | 0.4 ± 0.1     | 0.5 ± 0.2      | 2.2 ± 0.15      | 97 ± 0.5         |
| 1f         | 2.2 ± 0.06    | 8.5 ± 0.07     | 0.12 ± 0.05     | 89 ± 0.6         |
| 1a         | 1.7 ± 0.2     | 12.6 ± 0.15    | 2.4 ± 0.05      | 83.5 ± 0.2       |
| 2a         | 2.3 ± 0.14    | 14.9 ± 0.2     | 1 ± 0.05        | 81.7 ± 0.1       |

was seen (Annexin V labeling ~1.7–1.9% and 7-AAD staining ~6.4–11.4%) (Table 2). After 6 h, distraction process of cell membrane was progressed to 8.5–14.9% while apoptosis remained between 1.7–2.3% (Table 3, Figs. 2 and 3). This finding indicates that nitroheteroaryl-1,3,4-thiadiazole derivatives are membrane-attacking molecule for L. major promastigotes, suggesting that it acts directly on the plasma membrane, making it leaky and thus driving the programmed cell death signals.

3.3. Oligonucleosomal-DNA fragmentation mediated by nitroheteroaryl-1,3,4-thiadiazole derivatives in L. major

For further characterization of the changes mediated by 1f, 1a and 2a compounds in the nuclear material of promastigotes during cell death, TUNEL assay was performed to detect the free ends of DNA fragments. Oligonucleosomal-DNA fragmentation analysis of promastigotes treated with IC50 compounds for 4 h, showed clear oligonucleosomal fragmentation of genomic DNA, an explanation for cell death (Fig. 4). The extensive TUNEL-positive cells with excessive DNA breakage after short period of exposure indicated severe damage leading to rapid decision of the cells for necrosis-like death as the only escape pathway.

3.4. Detection of PARP-like protein cleavage during thiadiazole compounds-mediated cell death

Western blots analysis was performed in an attempt to identify the presence of PARP-like protein (MW ~78–80 kDa). Interestingly, a distinct cleavage of a PARP-like protein (MW ~64–kDa) was observed over a period of time, whereas cleavage of PARP-like protein did not appeared in the untreated control cells compare to drug-treated cells (Fig. 5).
4. Discussion

There are several reports of in vitro development of two growth stages of virulent *Leishmania* promastigotes. In the logarithmic growth phase, parasites have a low virulence but high growth rate; in the later stationary growth phase, parasites exert a high disease-developing potential but low growth rate (Laskay et al., 2008; van et al., 2006; Wanderley et al., 2006). Acid phosphatase of *Leishmania* form large structures of proteophosphoglycan with molecular weight of about 110–130 kDa which are secreted outside the cell, anchored in the plasma membrane through glycosyl phosphatidylinositol or kept inside the cell. This enzyme is one of the most important factors contributing to the spread of the virulent parasites and the infection in host cells. Acid phosphatase is produced and secreted in both stages of the parasite growth but the expression and activity of the enzyme is much higher in the stationary phase which is important in the establishment of the disease in this phase. The part of the enzyme placed on the surface, involved in adhesion of the parasite to host cells and infecting the cells. The portion of acid phosphatase secreted into the phagosome produces inorganic phosphate as a source of nutrition and disables hydrolys and is essential for survival of the parasite. It has been proven that these enzymes penetrate into the cytoplasm of immune cells and disrupt signaling pathway-dependent phosphorylation of proteins involved in production of active nitrogen radicals and cytokines. In addition, it disrupts interferon-γ receptor signaling and prevents the formation of oxidative complex NADPH and the oxidative burst in phagosome (Olivier et al., 2005). We showed that 1,3,4-thiadiazole derivatives exhibits potent anti-leishmanial activity on both parasite growth stages and decreases cell viability and specific activity of acid phosphatase (data not shown). These effects were more potent against the stationary phase. We do not know the compounds act directly or indirectly impacts on acid phosphatase activity.

It is known that induction of apoptosis in *Leishmania* parasite favors its silent entrance without induction of pro-inflammatory response (Arnoult et al., 2002; Laskay et al., 2008; Sen et al., 2004; van et al., 2006; Wanderley et al., 2006; Zangger et al., 2002) in contrast to induction of a necrosis-like death which stimulates elimination of invasive parasites (Arnoult et al., 2002; Elmore, 2007; Festjens et al., 2006; Zangger et al., 2002).

In order to clarify the modes of nitroheteroaryl-1,3,4-thiadiazole derivatives induced cell death during different incubation periods, Annexin V detection was performed at 3 and 6 h to show whether necrosis or apoptosis was induced. The study showed that necrosis (7AAD incorporation) was induced and increasingly up-regulated. The incubation time was short to avoid loss of promastigotes due to lysis which makes it impossible to perform apoptosis/necrosis flow cytometry. Therefore, percentage of necrosis was significantly increased but did not reach the highest level. Interestingly, the extensive TUNEL-positive cells with excessive DNA breakage were observed after 4 h of
treatment. Also, cleavage of PARP-like protein was demonstrated at 24 h.

Apoptosis and necrosis are programmed cell death and suicidal pathway which are extremely important in the development of mammals (Arnoult et al., 2002; Zangger et al., 2002). Programmed cell death has also a functional role in the biology of protozoan parasites such that features of programmed cell death pathways similar to those in multicellular organisms have been reported in the Leishmania (Arnoult et al., 2002; Sen et al., 2004; van et al., 2006; Zangger et al., 2002). Increasing evidences support the existence of caspase-independent cell death in higher eukaryotes and also in Leishmania, which provides backup suicide mechanism, a necrosis-like death if the classical apoptosis machinery fails or does not exist (Festjens et al., 2006; Fiers et al., 1999; Leist and Jaattela, 2001). Necrosis is characterized by irreversible disruption of plasma membrane integrity, organelles breakdown such as lysosomes and randomly-fragmented DNA. In necrosis, the cellular contents leak into the extracellular environment, whereby they may act as a “danger signal” to trigger macrophage activation and the concomitant induction of proinflammatory cytokine production (Brouckaert et al., 2004; Cocco and Ucker, 2001). Although the mechanisms and morphologies of apoptosis and necrosis are different, there is overlap between these two processes. Necrosis is considered to be a toxic process in which the cell is a passive victim and follows an energy-independent mode of death (Fiers et al., 1999; Leist and Jaattela, 2001).

However, reports about the pathways and the proteins implicated in the potential programmed cell death process in the unicellular Leishmania are very variable (Arnoult et al., 2002; Das et al., 2001; Sen et al., 2004; Sereno et al., 2001; Verma and Dey, 2004; Zangger et al., 2002). Apoptosis-like changes have been reported for Leishmania sp. in response to some agents such as oxygen radical species (Das et al., 2001), antimonial drugs including pentostam and glucantim (IC_{50} > 10 mg/ml for promastigotes and 30–50 μg/ml for amastigotes) (Croft and Yardley, 2002; Sereno et al., 2001), miltefosine (IC_{50}~25 μM) (Paris et al., 2004; Verma and Dey, 2004), amphotericin B (IC_{50}~0.9–0.4 mg/ml) (Lee et al., 2002), and the protein kinase inhibitor drug staurosporine (Katakura and Kobayashi, 1988).

We previously reported that some of the nitroheteroaryl-1,3,4-thiadiazole derivatives with potent anti-leishmanial activity can

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**Fig. 4.** TUNEL assay of logarithmic phase L. major treated with DMSO (A) and IC_{50} dilutions of analogs 1f (B), 1a (C) and 2a (D) for 4 hours. The extent of DNA fragmentation was analyzed by flow cytometry.

**Fig. 5.** Detection of PARP-like protein in L. major. The nuclear extraction was performed after thiadiazol IC_{50} treatments for 24 h at 25 °C. DMSO-treated control (lane 1), drug-treated samples (Lanes 2–8 corresponding to 1f, 2f, 2g, 2b, 2u, 2u, 1a, respectively); the bands were immune blots with anti-human PARP rabbit antibody probe.
selectively inhibit L. major topoisomerase catalytic activity through formation of DNA-cleavage complexes (Poorrajab et al., 2009b).

In this study some futures of genotoxicity of the compounds such as DNA strand breaks and over-activation of PARP were seen which lead to depletion of NAD+ and profound ATP. Depletion of cellular NAD+/ATP resources reduces cellular reduction capacity and energy failure followed by mitochondria over-activation and increased reactive oxygen species production/oxidative stress, which can result in disruption of membranes integrity and triggering necrosis in a caspase-independent cell death pathway (Das et al., 2006; Debrabant et al., 2003; Elmore, 2007; Festjens et al., 2006; Fiers et al., 1999; Lee et al., 2002; Leist and Jaattela, 2001; Proskuryakov et al., 2003). We also investigate the presence of PARP protein as one of the most important DNA enzymes. As a result, cleavage of PARP-like protein was observed implying the presence and functional role of this protein through the cell death in the kinetoplastid protozoan parasite L. major. Considering that L. major is the most ancestral unicellular eukaryote and its phylogenetic divergence predate by several hundred million years; this finding demonstrates the emergence of the protein and its conservation through a process of convergent evolution (Besteiro et al., 2007; Proskuryakov et al., 2003).

In conclusion, nitroheteroaryl-1,3,4-thiadiazole derivatives induced parasite killing without induction of apoptosis. These potent compounds also affect the acid phosphatase as the most important virulence factor; therefore in vivo efficacy, specificity and cytotoxicity of the compounds should be investigated.

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Reference